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Exercise protocol and muscular fiber type composition dependent phosphocreatine recovery in health and disease; a preliminary study

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Summary

^{31}P magnetic resonance spectroscopy provides tools to obtain bioenergetic data from skeletal muscle during exercise and recovery. The time constant (TC) of phosphocreatine (PCr) recovery is used as a measure of mitochondrial function. Muscle fibers are either slow twitch (ST) oxidative or fast twitch (FT) glycolytic fibers. ST fibers are generally recruited at lower force levels than FT fibers. However, at high contraction-velocities both types of fibers are recruited already at low force levels. This differential fiber type recruitment legitimates the distinction between exercise protocols of both low and increasing intensity at low contraction frequency (LE), and exercise protocols of high intensity and/or high contraction frequency (HE). Utilizing LE protocols, patients with mitochondrial encephalomyopathies (ME) and patients with migraine with aura (MA) have been demonstrated to differ from healthy volunteers (HV) with respect to TC values normalized for minimum pH reached during recovery (pH_{min}). Using an HE protocol it was not possible to detect the differences between controls and ME by means of ^{31}P-MRS. This result might be explained by higher fractions of activated FT fibers and interindividual variations in muscle fiber type composition in HV. Thus, our results may imply that the differences detected with LE protocols could result from activation of higher fractions of FT fibers in patients, rather than from comparing whole muscle oxidative capacities. This new finding needs to be verified by measuring both LE and HE protocols for each subject in a follow-up study.

Key words

^{31}P-MRS exercise protocol, muscular fiber type composition, contraction-velocity dependence, differential fiber type recruitment, workload independence, mitochondrial function, mitochondrial encephalomyopathies, migraine with aura.

Abbreviations

ATP_{tot}, total ATP signal; AUC_{PCr}, area under the curve of the PCr signal; CoQ_{10}, Coenzyme Q_{10}; FID, free induction decay; FT, fast twitch; HE, exercise of high intensity and/or high contraction frequency; HV, healthy volunteers; LE, exercise of low and increasing intensity at low contraction frequency; MA, migraine with aura; ME, mitochondrial encephalomyopathies; MELAS, Mitochondrial Encephalomyopathy, Lactic Acidosis, Stroke-like episodes syndrome; mtDNA, mitochondrial DNA; NARP, neuropathy, ataxia, retinitis pigmentosa syndrome; PCr, phosphocreatine; pH_{min}, minimum pH during recovery; P_i, free phosphate; ^{31}P-MRS, Phosphorus magnetic resonance spectroscopy; P_{tot}, sum of all recorded phosphorus signals; ST, slow twitch; TC, time constant.

1. Introduction

Phosphorus magnetic resonance spectroscopy (^{31}P-MRS) offers the possibility to study cell energy metabolism in vivo in various tissues. Due to the ability to perform exercise inside the MR scanner during data acquisition, ^{31}P-MRS has been used to study various muscle disorders for almost 30 years, mitochondrial cytopathies being amongst them (1).

Mitochondrial cytopathies are a diverse group of inherited and acquired disorders displaying inadequate energy production of the cell. Several syndromes linked to specific defects in the mitochondrial and nuclear genome have been identified. Mitochondrial encephalomyopathies (ME) are a subgroup of mitochondrial cytopathies in which both brain and muscle tissues are affected by the disease. In these patients migraine with aura is a common symptom. In general, migraine is a primary headache disorder which can be divided into the two subgroups: migraine with aura (MA) and migraine without aura.

By means of ^{31}P-MRS, ME and MA patients have been shown to differ from healthy controls with respect to recovery of the phosphocreatine (PCr) concentration in muscle tissue after exercise, which is represented by the time constant (TC) of the monoexponential recovery curve fit of the PCr peak as measured by ^{31}P-MRS (2-6), called recovery time. This difference is reflecting mitochondrial defects in ME patients since it is well accepted that PCr recovery after exercise is solely governed by aerobic metabolism (7,8).

To determine the maximal mitochondrial capacity to produce ATP, it has been pointed out that monitoring the recovery rate of PCr has the practical advantage that no measurement of force or performed work is required if the lowest pH value is reached during the first minute of recovery (termed minimum pH, pH_{min}) and

ranges between pH 6.75 and pH 6.95 (9-12). The known inhibitory influence of acidosis on creatine kinase elicits a direct linear relationship between recovery kinetics of PCr and pH values reached at the end of exercise. This relationship was claimed to be independent of either work output or the differential recruitment of glycolytic and oxidative fibers (12). Since the PCr signal recovery represents the average response of all fibers within sensitivity of the ^{31}P-MRS measurement (11,13), all fibers need to be recruited equally and maximally which is well accepted to be verified by pH_{min} values below 6.95 (9). It was proposed that when monitoring PCr recovery exercise protocols, eliciting pH values below 6.75 should be avoided due to non-monoexponential recovery (10,14). This is in part supported by the finding that for pH values <6.50 monoexponential fits for PCr recovery and the resultant TC values are not accurate (15).
However, these findings are in conflict with the reported substantial intersubject differences in the effect of acidosis on PCr recovery kinetics (16) and with the reported dependence of oxidative recovery on fiber recruitment (13), which in turn depends on the exercise protocol employed.

Basically, muscle fibers are divided into two types, slow twitch (ST) fibers and fast twitch (FT) fibers. In general ST fibers are more oxidative with higher PCr resynthesis rates and they have lower anaerobic potential with less acidification in response to exercise than FT fibers. All muscle fiber types exhibit contraction-velocity dependent recruitment behavior: the higher the contraction-velocity the lower the force levels at which they are activated; and at a given force level only motor units reaching their tonic threshold (defined as the minimal steady-force level maintaining a steady discharge of the motor unit) remain active during sustained contraction (17,18). ST fibers are recruited and stay active at lower force levels than FT fibers. Interindividually there are substantial variations in fiber type compositions of muscles (e.g. gastrocnemius muscle) (18).

During protocols of low intensity exercise and of increasing intensity exercise (ramp protocols) at low contraction frequency (LE) ST fibers are mainly recruited. With increasing workload at low contraction frequency the fraction of recruited FT fibers will increase. Still only part of them will remain active throughout a sustained contraction. Therefore, when PCr has dropped enough to assess its recovery rate in a LE protocol, full FT fiber activation, if at all, will have only been maintained for a comparatively short period and the PCr drop observed at the end of exercise will essentially result from ST fibers. Since FT fiber activation is mainly responsible for pH changes within the sensitivity volume of the receiving coil, pH will most likely have dropped less at a comparable PCr drop in LE than in protocols of high intensity and/or high contraction frequency exercises (HE). Hence, recovery rates after LE can be regarded as the oxidative capacity of ST fibers rather than as the oxidative capacity of the entire muscle under investigation. HE protocols on the other hand have a higher probability of activating all fiber types more or less equally right from the start of exercise and therefore show a more substantial drop in pH as a result of the overall greater FT fiber involvement (19). This hypothesis is supported by the fact that with HE protocols line broadening or even splitting of the resonance line of free phosphate (P_i) can be observed in ^{31}P-MRS since the chemical shift of P_i is pH dependent. P_i splitting indicates two compartments with different pH; most likely ST and FT fibers, but is said to only occur at exhaustive work levels (20). Consequently, with HE protocols a PCr recovery rate composed of a mixture of recovery rates from ST and FT fibers will result, giving a better approximation of whole muscle oxidative capacity. The mixed answer regarding PCr recovery will strongly depend on the individual differential muscle fiber type composition.

From the above considerations we hypothesize, in line with the findings mentioned above (13), that the dependence of differential fiber type recruitment on contraction speed and exercise intensity may produce different pH_{min} values and TC values

contingent on the employed exercise protocol in cooperation with the individual fiber type composition of the muscle under investigation. Thus, it might be further hypothesized that the difference in muscle fiber composition and recruitment as well as that in proton efflux rates - influencing the pH_{min} and known to be higher in ME patients (21) - between healthy volunteers (HV), MA and ME patients might mask the mitochondrial impairment in the patient groups when using a HE protocol to investigate muscle energy metabolism with in vivo ^{31}P-MRS. Therefore it can be assumed that training, which changes muscle fiber type composition, might have an influence on the determined TC and pH_{min} values. In addition oxidative capacity has been revealed to decrease with age by 50% as was reported for elderly (65-80 years) healthy subjects (22).

Hence, our preliminary study intends to investigate the influence of the employed HE protocol and thus of fiber recruitment on PCr recovery rates and its interrelation to the minimal pH. A high contraction-velocity submaximal exercise protocol was implemented in order to fully activate all muscle fibers investigated and was applied to HV, ME patients and MA patients. By limiting the exercise duration to two minutes, pH was expected neither to fall too extensively nor to show compartmentation, since P_i splitting is indicative of strenuous exercise which can mimic disturbed mitochondrial function (23). Within two minutes sufficient blood flow and more importantly O_2 delivery adjustments were allowed for (24-26) and tolerable loads were attained to show sufficient [PCr]-depletion to asses the TC, even for the clinically most severely affected ME patients.

By comparing patient data to four different subgroups of HV (for details see section 3.4. Data analysis) the influence of the confounding factors age, endurance training, strenuous exercise (as individually experienced) and pH_{min} 6.50 as lower limit for monoexponential PCr recovery were examined.

2. Methods

2.1. Controls and patients

25 healthy volunteers (HV), 11 migraine patients with aura (MA) and 9 patients with mitochondrial encephalomyopathy (ME) were investigated. After applying the quality criteria (explained in section 3.4. Data analysis) to the recorded data, 21 HV (12 females, 9 males; age: 18-64 years, average: 36.5 years), 5 MA (4 females, 1 male; age: 18-37 years, average: 25.2 years) and 6 ME (3 females, 3 males; age: 28-58 years, average: 43.7 years) were included into the analysis. All examinations were carried out according to the standards set by the latest revision of the Declaration of Helsinki and the study protocol was approved by the local ethics committee Zürich. Written informed consent to participate in this study was obtained in all cases. Migraine patients were diagnosed clinically according to the second edition of the International Classification of Headache Disorders (ICHD-II) (27). MA were free from headache attacks for at least ± 3 days around the date of examination and did not take any preventive or attack medication at that time. Diagnoses of mitochondrial cytopathies following international criteria were confirmed by either pathological findings and/or molecular genetic analysis. Four ME patients received daily oral Coenzyme Q_{10} (CoQ_{10}) therapy (3 x 100 mg). Even under CoQ_{10} medication these four ME patients were clinically severely handicapped and three of them showed heavy impairment of muscular performance such as difficulties in building up the required pressure values and quick fatigue. Table 1 additionally lists other medications. Patient ME1 is carrier of the A3243G MELAS-mutation (Mitochondrial Encephalomyopathy, Lactic Acidosis, Stroke-like episodes syndrome) but apart from migraine headaches has not become clinically

manifest so far. Patient ME2 has the neuropathy, ataxia, retinitis pigmentosa (NARP) syndrome but had no additional muscular symptoms.

Table 1
ME patients

ME	diagnostic method	mutation	clinical presentation	CoQ10	other medication
1	Molecular genetic	mtDNA A3243G	Asymptomatic	no	Oral contraceptive
2	Molecular genetic	mtDNA T8993G	NARP	yes	Riboflavin
3	Molecular genetic	mtDNA C12346T	Optic neuropathy, Myopathy	yes	Riboflavin
4	Molecular genetic	mtDNA T7679C (F32L)	Chorea syndrome	yes	Clonazepame
5	Bioptic	n.d.	PEO, Myopathy	no	no
6	Bioptic	n.d.	Cerebellar ataxia, PEO, Sensorineural deafness, Encephalopathy	yes	Clonazepame, Amitriptyline, Citalopram, Esomeprazole, Alendronate, Calcium carbonate, Cholecalciferol, Perindoprile, Ibuprofene, Transdermal fentanyl

PEO: Progressive external ophthalmoplegia
NARP: Neuropathy, Ataxia, Retinitis pigmentosa
n.d. not determined

Table 1: Methods used to validate the diagnosis of mitochondrial cytopathies, mutations found within the mitochondrial genome and medication at the time of investigation.

2.2. Exercise protocol

In-magnet exercise was performed by pressing a pedal with the right foot. Resistance of the pedal was given by a blood pressure cuff initially filled up to 20 mmHg. The subject being examined was asked to press the pedal at a frequency of 80/min (28), triggered by a metronome, and to build up pressure values of 80 to 150 mmHg with every tread. The plantar flexions were executed as rapidly as possible. A manometer served to control the pressure built up and the tread frequency. Oral feedback from the examinator was given to the subject.

To avoid movement, the leg performing the exercise was fastened to the examination table just above the knee and the respective foot was attached to the pedal. The subject under examination held two straps, one in each hand in order to maintain the position on the examination table.

2.3. ^{31}P magnetic resonance spectroscopy

^{31}P magnetic resonance spectroscopy (^{31}P-MRS) measurements were acquired on a whole-body 1.5 T MR system (Gyroscan Intera; Philips Healthcare, Best, the Netherlands) with a 10 cm diameter single-tuned circular transmit/receive surface coil positioned on the right calf muscle using a pulse-and-acquire technique. This resulted in a measured volume of roughly 260 ml in the shape of a hemisphere centered at the center of the coil. Excitation was carried out by a BIR-4 (29) (B1 insensitive rotation) pulse set to a flip angle of 40 degrees, using a receiver bandwidth of 1500 Hz. A time series of 100 free induction decays (FIDs) with 512 samples per FID was recorded, using the following 10 min paradigm:

2 min (20 spectra) resting phase, 2 min (20 spectra) exercise phase, 6 min (60 spectra) recovery phase. Each time step consisted of 4 averages with a repetition time of 1.5 s each. The shim volume was chosen to fit the anatomy of the subject and was roughly 80 ml (ranging from 35·35·60 mm^3 = 73.5 ml to 35·40·65 mm^3 = 91 ml) (Figure 1).

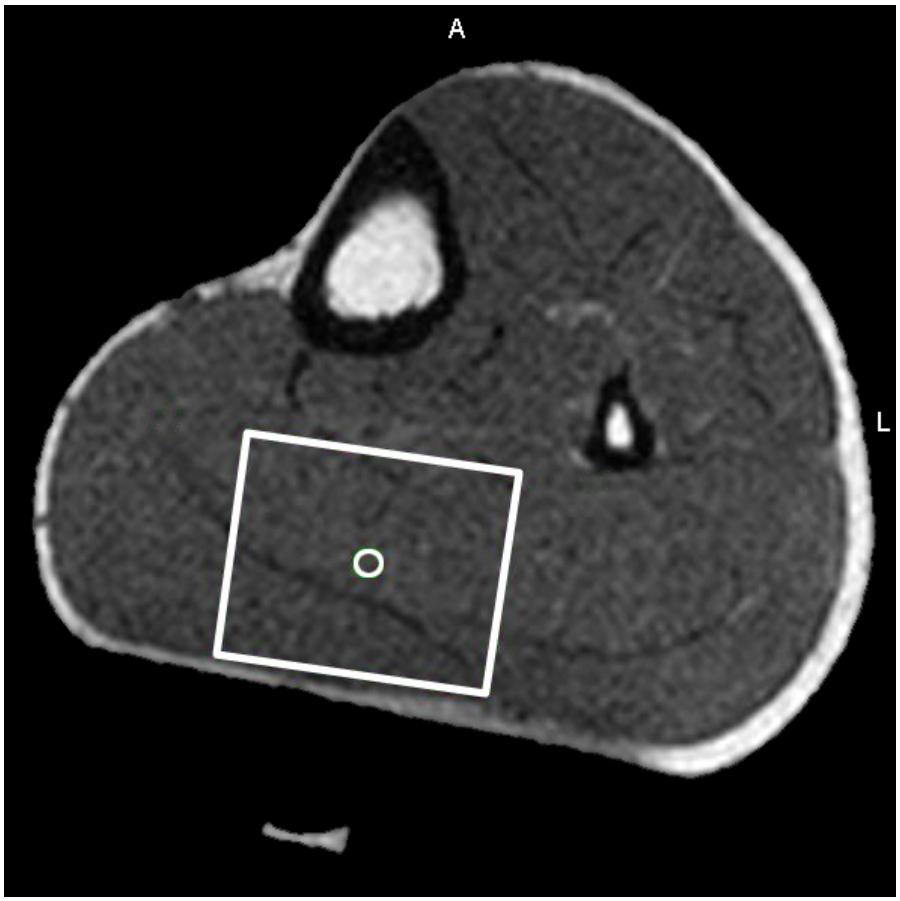

Figure 1: Planscan of the right lower leg demonstrating the chosen shim volume. The water phantom within the surface coil shows the center of the coil relative to the shim volume. A: anterior; L: left.

2.4. Data analysis

Postprocessing of the spectra was done using the jMRUI v2.2. software package (30). Zero order phase correction was applied manually and a 9.8 Hz apodization filter and DC (direct current) correction using the last 150 points of the FID were executed on the time series.

The AMARES algorithm (31) within jMRUI provided the means for quantification. Table 2 lists starting values for prior knowledge (32) on chemical shift, coupling constants and line shapes used for fitting. All signals were modelled as Lorentzian functions, and the amplitudes of the ATP doublets (α- and γ-ATP) and triplet (β-ATP) were fitted with the fixed ratios 1:1 and 1:2:1 respectively. Soft constraints applied on chemical shift and line width are given in Table 3.

Table 2
starting values for fitting

peak	frequency (Hz)	line width (Hz)
α-ATP_1	-203.4	9.1
α-ATP_2	-185.0	
β-ATP_1	-428.0	6.1
β-ATP_2	-420.5	
β-ATP_3	-403.7	
γ-ATP_1	-71.9	9.1
γ-ATP_2	-58.1	
PCr	0.0	9.1
P_i	123.7	9.1

Table 2: Starting values for fitting

Table 3

prior knowledge - soft constraints for fitting

peak	frequency (Hz)	line width (Hz)
α-ATP_1	-211.9 – -193.9	0.0 – 30.0
α-ATP_2	fs: +19.25	
β-ATP_1	fs: -11.47	0.0 – 30.0
β-ATP_2	-429.1 – -411.0	
β-ATP_3	fs: +11.47	
γ-ATP_1	-80.1 – -62.0	0.0 – 30.0
γ-ATP_2	fs: +15.86	
PCr	estimated	0.0 – 40.0
P_i	103.4 – 129.2	0.0 – 25.0

fs = fixed shift, j-coupling constant between the multiplet peaks

Table 3: Prior knowledge for fitting

The manually applied zero order phase correction was fixed and the first order phase correction was kept to zero for quantification. Recovery of the PCr signal after exercise is described by the recovery time TC of the monoexponential function

$$s = a \cdot \left(1 - d \cdot e^{\frac{-t}{TC}}\right), \qquad \text{(eq. 1)}$$

with: s : calculated area under the curve of the PCr signal (AUC_{PCr}) in a single spectrum at time t

a : largest AUC_{PCr} reached at the end of recovery (last spectrum)

d : difference in % between a and the lowest AUC_{PCr} at the beginning of recovery (first spectrum)

e : Euler's number

t : time in seconds after start of time series at the end of recording the respective single spectrum; discrete time steps of 6 seconds

A best fit to the experimental points (9) was calculated by adjusting the variables TC, a and d by using the program Solver in Excel 2002 (SP-2, Microsoft Corporation, Redmond, WA).

Rigorous quality criteria were applied to the spectral time series. Single spectra within the time series were eliminated if all of the following three parameters deviated more than 5 % from their average over the whole time series: total ATP signal (sum of α-, β- and γ-ATP signals; ATP_{tot}), the sum of all recorded signals (P_{tot}) (9,12) and the noise from the residue of the fitted signal. These three parameters should remain more or less constant throughout the entire experiment (12) if scan and fit are of high quality. Experiments with more than 10 % of the

single spectra in the recovery phase (6 spectra) not meeting these criteria were excluded from the study. Furthermore, experiments were excluded if the exercise and/or the recovery phase differed more than 6 s from the protocol owing to insufficient compliance of the investigated subject. Altogether the experimental data of 13 subjects (4 HV, 6 MA and 3 ME) were excluded from the study. pH values were calculated by jMRUI with the following modified Henderson-Hasselbalch equation:

$$pH = pK_a + \log_{10}\left[\frac{\delta_{obs} - 3.275}{5.685 - \delta_{obs}}\right], \qquad \text{(eq. 2)}$$

with: pK_a : = 6.73; negative common logarithm of the acid dissociation constant K_a

δ_{obs} : chemical shift observed between P_i and PCr in parts per million (ppm)

Statistical analysis included checking the data for normal distribution, quantifying the differences between the groups with the effect size index δ (33) and testing for significance with ANOVA using the SPSS 17.0 software (SPSS Inc., Chicago, IL). Linear regression analysis (SPSS 17.0) was used to model the relationship between the TC value and pH_{min}.

Patient data was then compared to four different subgroups of HV: (i) all, (ii) HV that were younger than 60 years (22), non-endurance trained (18) and who reached near steady state levels of PCr at the end of exercise (23), (iii) HV with pH_{min} above pH 6.50 (15), and (iv) HV of subgroup (ii) with pH_{min} above pH 6.50.

3. Results

All patients and controls reached a sufficient extent of PCr depletion (>5% of resting [PCr]) to assess the rate of PCr resynthesis during recovery (14). Two ME patients were not able to reach the specified minimum pressure of 80 mmHg throughout the entire exercise and 4 ME, 2 MA and 7 HV did not work at the specified frequency of 80/min (range: 30-90/min) during the entire experiment. PCr depletion ranged from 12-87% (ME), 24-68% (MA) and 17-80% (HV). Visual inspection suggested near steady state values at the end of exercise in all ME, 4 of 5 MA and 18 of 21 HV. ATP homeostasis and constancy of P_{tot} was maintained throughout the exercise protocol. During exercise P_i splitting in 1 MA patient and 4 HV and P_i line broadening in all investigated subjects was observed. P_i line widths increased by factors ranging from 1.4-3.7 (ME), 2.0-3.0 (MA) and 1.5-3.6 (HV). These findings confirm the more likely submaximal than intense character of the employed exercise protocol (10,34).

Typical single spectra of gastrocnemius muscle in the resting phase and at the end of exercise are shown in Figures 2a and 2b, respectively. The typical recovery pattern of the experimental PCr signal and the corresponding monoexponential fit are both displayed in Figure 3.

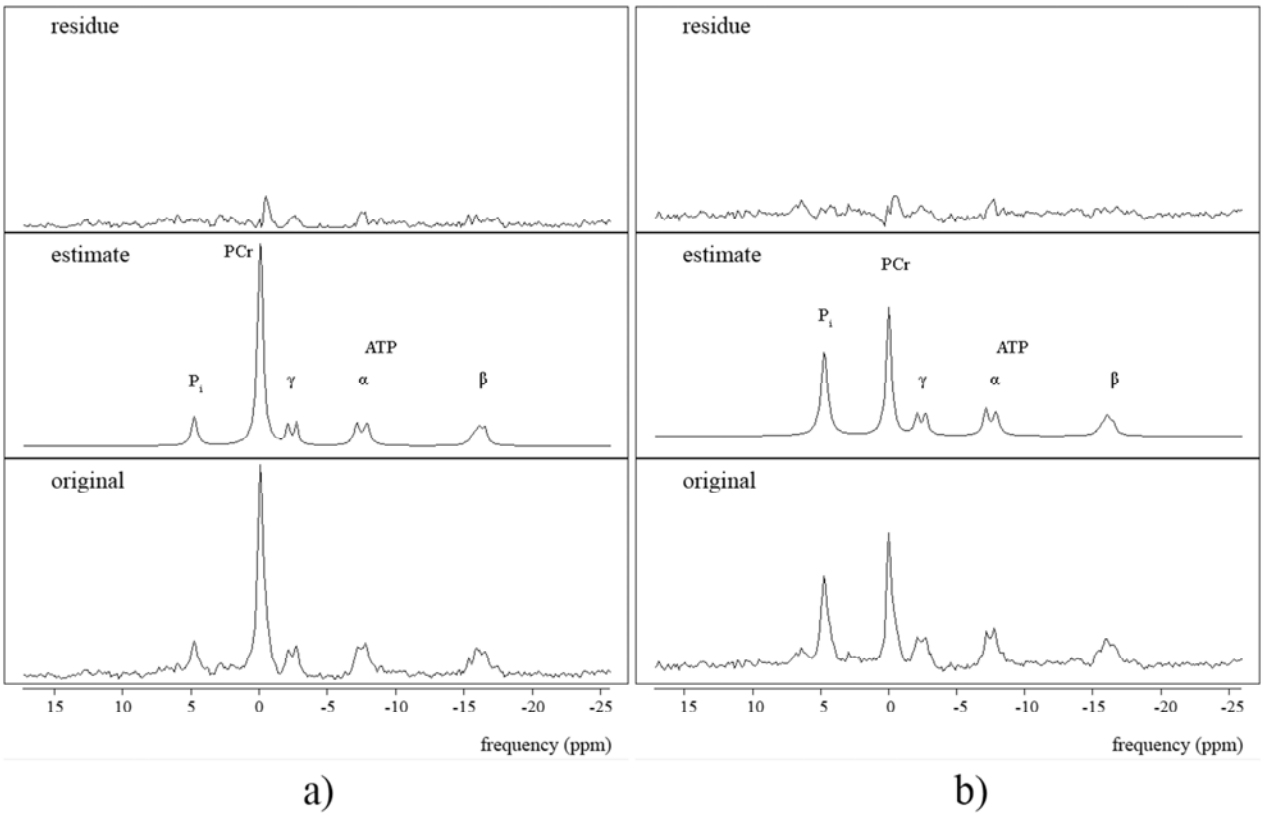

Figure 2: Typical single spectrum of gastrocnemius muscle:

a) in the resting phase;

b) at the end of exercise (last spectrum of the working muscle in the exercise phase);

The bottom panel shows the original spectra before fitting with the jMRUI, v2.2. software package the center panel shows the estimate of the signals after fitting and the top panel displays the residues of the original signals after subtraction of the estimated signals. Signal intensities are in arbitrary units and have been scaled to a comparable level with respect to the ATP signals, which remain at a constant value throughout the entire examination. Frequencies are reported in parts per million (ppm).

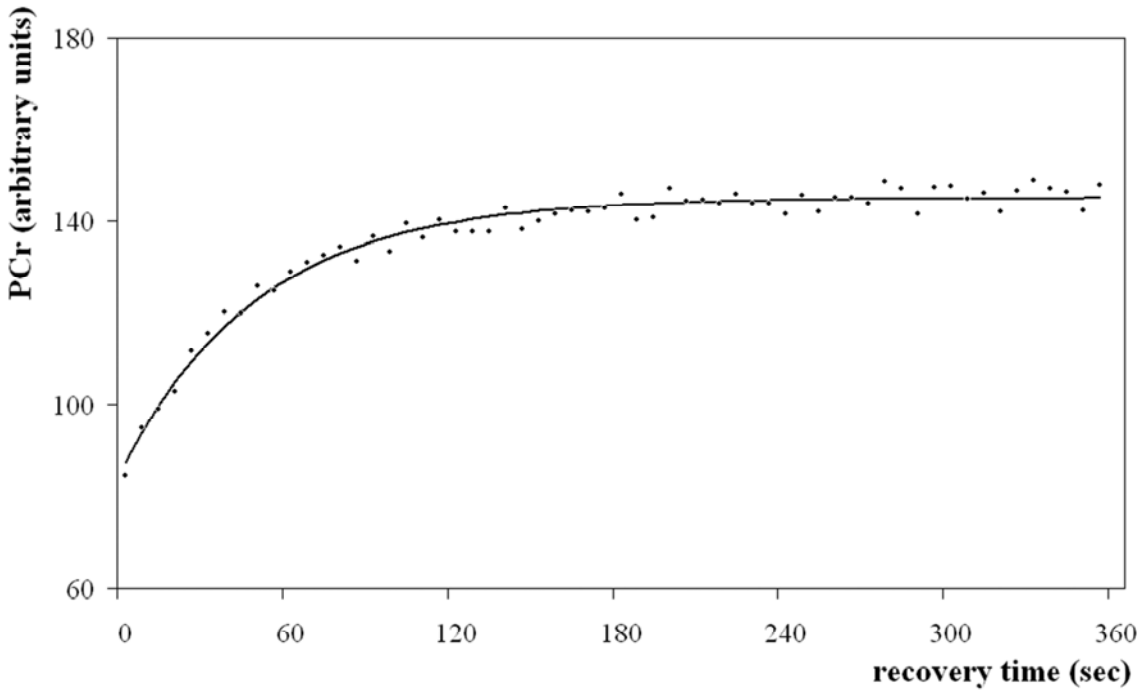

Figure 3: Typical recovery pattern of the phosphocreatine (PCr) signal after exercise in a healthy volunteer. The experimental PCr signals are shown as black points at intervals of 6 seconds. The calculated monoexponential fit of these recorded data points is indicated as black line.

Minimum pH ranged from pH 6.47-6.92 (ME), pH 6.42-6.95 (MA) and pH 6.39-6.94 (HV), thus staying ≤ pH 6.95 in all cases. TC values extracted from the monoexponential fits plotted against pH_{min} are reported for all subjects in Figure 4a. All data, including patient data, lie within the 95% individual prediction interval of the regression line ($y=-43\cdot x+329$; correlation coefficient $r=-0.56$) linking the TC values of PCr recovery to the pH_{min} values of the HV. Regression analysis after excluding seven HV data sets - three HV older than 60 years, one HV who did not reach near steady state level of PCr at the end of exercise and three HV who were endurance trained - resulted in two ME data sets lying outside of the 95% individual prediction interval of the regression line ($y=-20\cdot x+168$, $r=-0.40$) and another ME data set at the border of the 95% individual prediction interval (Figure 4b). Additionally, taking into account the non-monoexponential recovery of PCr at pH <6.50 regression analysis was performed for data sets with pH >6.50. A correlation coefficient r of −0.77 was found for the HV after the above mentioned exclusions. Two ME and one MA data point lie outside of the 95% individual prediction interval of the regression line ($y=-99\cdot x+710$). For comparison regression analysis was also performed for pH_{min} >6.50 without exclusion of any HV data sets. One HV and one ME data point lie outside of the 95% individual prediction interval of the regression line ($y=-113\cdot x+809$, $r=-0.71$) (Figures 4c and 4d). Interestingly the three data points of the endurance trained HV are highly linearly correlated, $r=-0.99$, with the following equation for the regression line, $y=-44\cdot x+323$ (plot not shown).

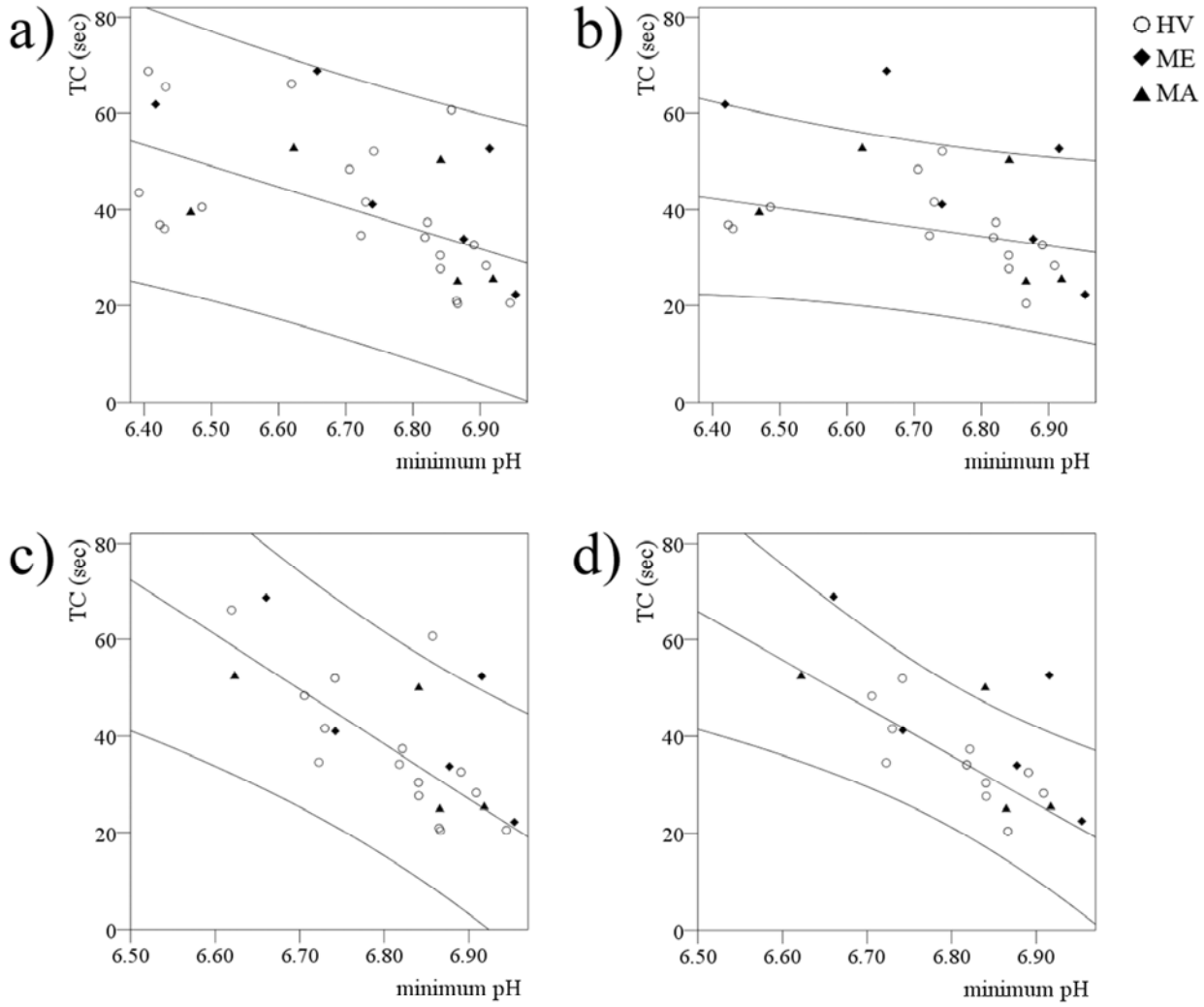

Figure 4: Phosphocreatine (PCr) recovery time plotted against minimum pH (pH_{min}) *without exclusions* in a) and c) compared to *with exclusions* in b) and d); equation for regression line and r value;

a) 21 healthy volunteers (HV; open circles), 6 patients with mitochondrial encephalomyopathy (ME; black diamonds) and 5 migraine patients with aura (MA; black triangles); $y=-43 \cdot x+329$, $r=-0.56$; all recorded data lie within the 95% individual prediction interval of HV;

b) after exclusion of 7 HV: 3 older than 60 years, 1 that did not reach near steady state levels of PCr at the end of exercise (as 2 of the HV older than 60 years also did not) and 3 that were endurance trained: 14 HV, 6 ME, 5 MA; $y=-20\cdot x+168$, $r=-0.40$; 2 ME data sets lying outside and 1 ME data set lying at the border of the 95% individual prediction interval of HV;

c) pH_{min} >6.50: 15 HV, 5 ME, 4 MA; $y=-113\cdot x+809$, $r=-0.71$; 1 HV and 1 ME data point lie outside of the 95% individual prediction interval of HV;

d) pH_{min} >6.50: after exclusion of 4 HV: 1 older than 60 years, 1 that did not reach near steady state levels of PCr at the end of exercise and 2 that were endurance trained: 11 HV, 5 ME, 4 MA; $y=-99\cdot x+710$, $r=-0.77$; 2 ME and 1 MA data point lie outside of the 95% individual prediction interval of HV;

The PCr recovery time is represented by the time constant (TC) of the monoexponential function best fitting the experimental points and is plotted as a function of pH_{min} which refers to the lowest value of cytosolic pH reached during the first minute of recovery after exercise. The central straight line in each plot illustrates the linear regression linking the TC values to the pH_{min} values of the HV and the two outer lines delimit the corresponding 95% individual prediction interval.

The ^{31}P-MRS spectra of resting gastrocnemius muscle were examined for differences between groups in the ratios of P_i/PCr, which allows an estimate of the cytosolic free [ADP], PCr/ATP, and P_i/ATP. All three of these ratios represent an indicator of the energy status of the examined muscle tissue and have been reported to differ between groups of ME and HV by different investigators (10,21,28,35-38). In accordance with results of other research groups (1,3,5,10,39-43) the analyzed ratios in this study were not significantly different (Bonferroni adjusted p-values between 0.597 and 1.0) between the three groups (Figure 5a, 5b and 5c).

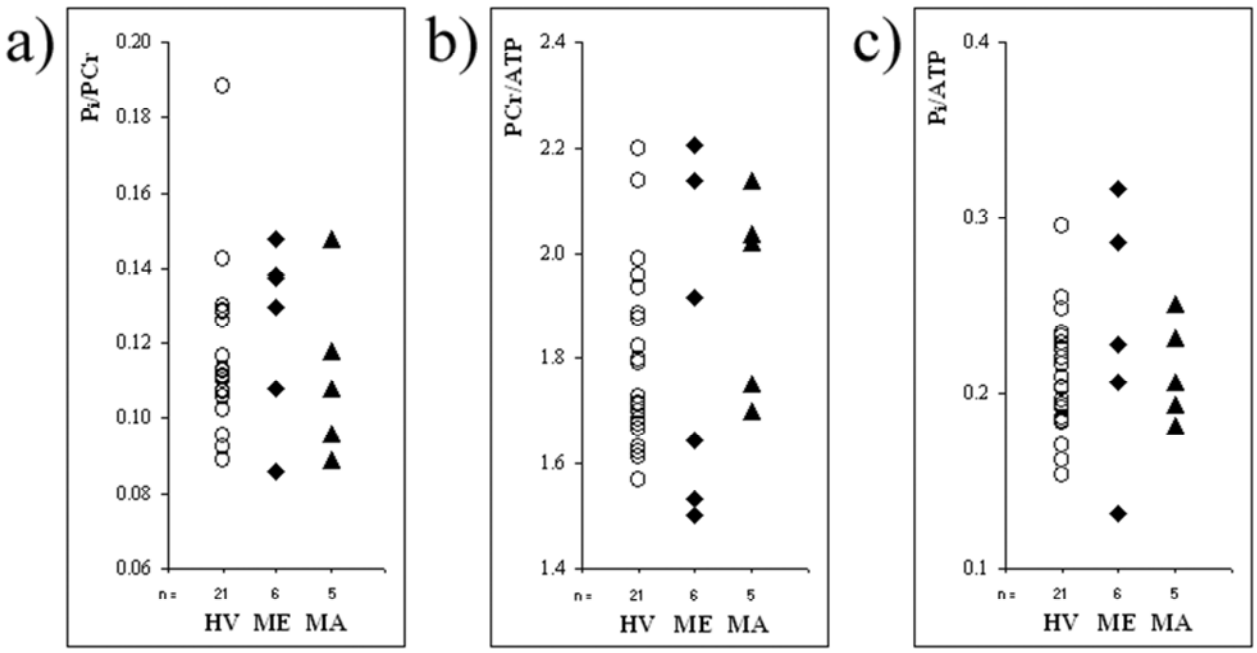

Figure 5: Scatterplots of resting gastrocnemius values of:

a) P_i/PCr-ratio;

b) PCr/ATP-ratio;

c) P_i/ATP-ratio;

for 21 healthy volunteers (HV; open circles), 6 patients with mitochondrial encephalomyopathy (ME; black diamonds) and 5 migraine patients with aura (MA; black triangles).

4. Discussion

To date it is believed that two parameters, the TC of PCr recovery and pH_{min}, give full information on the muscle's oxidative capacity: a pH_{min} below 6.95 is reported to indicate sufficient muscular activation to assess the full mitochondrial capacity to synthesize ATP by measuring TC (9,14), since it is well accepted that recovery after exercise is solely governed by aerobic metabolism (7,8). In our study, all patients and controls showed an adequate drop in [PCr] during exercise to reliably extract the TC of PCr recovery from the acquired data (14). Minimum pH was below or equal pH 6.95 in all volunteers and patients. However, as hypothesized in the introduction the employed HE protocol could not demonstrate the previously reported (2-6) differences between the investigated groups of ME, MA and HV (Figure 4a) although this study complied with the above requirements on pH_{min} and PCr drop. More precisely, according to those reports it was anticipated that all ME and at least some MA data points would come to lie outside the 95% individual prediction interval of the linear regression analysis of TC as a function of pH_{min}. In this study all patients lie inside the 95% prediction interval when it is derived from all HV.

The direct linear relationship between recovery kinetics of PCr and pH values reached at the end of exercise was claimed to be independent of either work output or the differential recruitment of glycolytic and oxidative fibers during low frequency isotonic exercise (of LE type) by investigating the forearm flexors of healthy subjects (12). By employing an isokinetic low frequency ramp exercise protocol (also of LE type) the linear relationship between TC and pH_{min} could also be demonstrated for calf muscles in HV ($y=-46\cdot x+342$, $r=-0.92$) (9).

With r=−0.92 a clearly different correlation coefficient was found by this latter group in comparison to this study (HE type) with r=−0.56. After excluding HV for reasons of age above 60 years, strenuous exercise as individually experienced, endurance training and pH_{min} values <6.50, the correlation coefficient in our control group data improved to give good linear correlation with r=−0.77 and a regression line equation of y=−99·x+710.

This improvement of linear correlation is in line with the hypotheses developed in the introduction. The following paragraphs discuss these aforementioned possible reasons behind this finding and possible explanations for the relationship between HV and patient data found in this study: (i) a greater spread in the control group due to age and training effects on muscle fiber composition and oxidative capacity, (ii) non-monoexponential PCr recovery for pH <6.50, (iii) differential muscle fiber type recruitment in HE and LE protocols and (iv) additional compensation mechanisms in ME and MA patients.

(i) By comparing younger adults (25 to 48 years) with elderly subjects (65 to 80 years), oxidative capacity has been revealed to decrease with age by up to 50% (22). Looking at our data the three HV older than 60 years had strikingly higher TC values compared to the younger HV with comparable pH_{min}. In addition two of the three HV older than 60 years did not reach near steady state PCr levels during exercise, had pH_{min} <6.50 and featured P_i splitting during exercise and recovery. Not reaching near steady state PCr levels during the employed HE protocol as well as P_i splitting is indicative of strenuous exercise as individually experienced (20). Such strenuous exercise in healthy subjects can mimic disturbed mitochondrial function (23), which is well in line with our data. A third HV (aged 18) did not reach near steady state PCr levels, also showed a remarkably higher TC value than expected and P_i splitting. We could not identify any reason for this behavior.

It is known that endurance training influences muscular oxidative capacity (44-46). Endurance trained have considerably higher fractions of ST fibers (18) and

therefore demonstrate relations between pH_{min} and TC values in our HE protocol as do sedentary HV in LE protocols, where mainly ST fibers are activated. This is demonstrated by good agreement of regression line equations for the endurance trained in our study ($y=-44 \cdot x+323$, $r=-0.99$) and sedentary healthy controls in the above mentioned isokinetic low frequency ramp exercise protocol study ($y=-46 \cdot x+342$, $r=-0.92$) (9). Three HV performed regular endurance training, of which one showed a pH_{min} <6.50 and a split P_i peak during exercise.

(ii) As stated in the introduction it was demonstrated that monoexponential fits of PCr recovery and the resultant TC values are not accurate for pH values <6.50 (15).

(iii) In contrast to LE protocols HE protocols have a higher probability of activating all fiber types more or less equally right from the start of exercise and therefore have a PCr recovery rate composed of a mixture of recovery rates from ST and FT fibers. Then the lower absolute r values found in this study compared to $r=-0.92$ of the above mentioned isokinetic low frequency ramp exercise protocol (LE; mainly ST fiber recovery) possibly reflect the interindividual variations in muscle fiber type composition detected with HE protocols. It is therefore reasonable to assume that the linear correlation of pH_{min} and TC is worsened and the slope of the regression line changes with diverse fractions of FT fibers involved in the PCr recovery. Hence both are strongly dependent on the individual fiber type compositions of the members of the investigated groups.
Only at pH_{min} >6.50 and when excluding the one HV >60 years of age and the two endurance trained HV is our control group homogeneous enough to give a high linear correlation ($r=-0.77$) and with it a 95% individual prediction interval, which does not contain two of five ME patients and one of three MA patients.

(iv) It was deduced earlier that HV display higher TC values in recovery from HE protocols than from LE protocols, whereas the difference between TC values for HE and LE protocols in ME patients is supposedly comparatively small. In order to comply with the demands of either exercise protocol ME patients are presumed to be forced to recruit FT fibers at an earlier stage than HV in any protocol and therefore have a lower dependency of TC values on exercise protocol because of a more or less exercise independent mixture of recovery rates from ST and FT fibers. In both protocol types ME patients should remain at higher pH levels than the HV (as reported previously (21) and shown for our HE protocol in Figure 4a) as a result of their increased proton elimination capacity; although anaerobic glycolysis is more active than in HV. Added up, these findings suggest that ME have approximately the same TC and pH_{min} values in LE and HE protocols, whereas HV do not. Thereafter, HV have lower TC and higher pH_{min} values in LE protocols than in HE protocols, which leads to the hypothesized masking of the mitochondrial impairment in ME patients with HE protocols. Altogether these features might explain the behavior of the ME patients data sets investigated in this study, since it has been reported, that by employing a ramp exercise protocol it should be possible to discriminate healthy and diseased muscles (with respect to functionality of mitochondria oxidation) with 100% specificity and sensibility even in the absence of any clinical symptoms and signs (4).

Mitochondrial cytopathies do not affect all muscles equally due to the pattern of inheritance or acquirement as well as propagation of the nuclear DNA and mitochondrial DNA (mtDNA) defects, respectively. Since there was no clinical indication no muscle biopsies of the investigated calf muscles were performed on ME patients within the framework of this study. Therefore, certainty of investigating muscles with defect mitochondria was not granted. Clinical symptoms of leg muscle weakness were the only available signs indicating infestation with defect mitochondria of the calf muscle. The two ME patients (ME4 and ME6, see also Table 1) identified in Figure 4d are both clinically severely affected with

severe involvement of the legs. Two of the three other ME patients (ME1 and ME2) either do not have any clinical symptoms apart from migraine headache (ME1) or do not have any muscle symptoms (ME2). In addition, ME1 regularly performed aerobic training. It is surprising that ME3, who shows heavy weakness in leg muscles, has her data point lying on the regression line for the HV in Figure 4d. ME3 had difficulties in reaching the specified workload, worked at a frequency of 40/min but showed a comparatively large drop in PCr (48% of the resting value) at that workload. ME4, ME6, ME2, and ME3 had been on CoQ_{10} therapy for more than 6 months at the time of investigation. Many studies have been performed on the effect of CoQ_{10} in mitochondrial cytopathies, some of them using ^{31}P-MRS, reporting controversial results concerning the effectiveness of CoQ_{10} in improving PCr recovery rates after exercise (6,28,47). One of those ^{31}P-MRS studies employed a ramp exercise protocol and plotted TC values as a function of pH_{min} and compared patients to HV. All ten patients were well outside the 95% confindence interval of the HV before CoQ_{10} treatment and all but two were still outside after CoQ_{10} treatment (6). ME4 and ME6 data points support the latter finding. ME3 could be like one of the two patients who showed normal PCr recovery after CoQ_{10} therapy or like the single therapy responder in one of the other reports, where a HE protocol (80 contractions/min) was used (28).

It had been demonstrated that some MA patients differ from healthy controls in their PCr recovery rates (2,5) or that some come to lie outside the normal range of TC values as functions of end exercise pH (3). All three cited studies employed steady state exercise protocols with stepwise increasing workloads (of LE type). MA patients do not feature increased proton efflux, as do ME patients, but it has been pointed out that they have a reduction in glycolytic flux and therefore less acidification at comparable PCr breakdown values (5). Using the same argumentation as above for ME patients, in MA patients this reduced glycolysis increases the fraction of activated FT fibers in LE protocols, since more FT fibers are needed to fulfill the same requirements imposed by the exercise protocol.

Consequently, LE protocols generate TC values composed of a mixture of ST and FT PCr recovery times in MA patients. In HE protocols the differences between MA and HV would therefore disappear in consequence of the more similar mixture of activated ST and FT fibers between groups.

For completeness, differences in resting values between groups were looked for. The ratios of P_i/PCr, PCr/ATP, and P_i/ATP have been reported to differ between groups of ME and HV by different research groups (10,21,28,35-38). In line with results of other investigators (1,3,5,10,39-43) the analyzed ratios in this study were not significantly different between the three groups (Figure 5a, 5b and 5c).

In conclusion, it was possible to emphasise the importance of exercise protocols and differential fiber recruitment when investigating diseased in comparison to healthy muscles. It seems possible that the differences between groups of healthy controls and ME patients observed in previous studies employing LE protocols on gastrocnemius muscle result from activation of higher fractions of FT fibers in muscles of ME patients, rather than from truly comparing whole muscle oxidative capacities. Thus, when employing HE protocols, higher fractions of activated FT fibers and the interindividual variations in muscle fiber type composition in HV could be masking the differences between controls and ME patients, which can be detected with LE protocols. Additionally, the differential fiber recruitment in dependence on exercise protocols challenges the notion of absolute workload independence of PCr recovery rates. All these findings need to be verified in follow-up studies where subjects have to be investigated with both LE and HE protocols and where exercise intensity has to be recorded.

Our study confirmed the confounding factors age, endurance training, strenuous exercise (as individually experienced) and pH_{min} 6.50 as lower limit for monoexponential PCr recovery and therefore requires the follow-up studies to match groups for age, sex and level of training and to exclude data sets with pH_{min}

<6.50. Also, for certainty of investigating diseased/unaffected muscle in ME patients, non-ME patients respectively, biopsies of the investigated muscle need to be performed on each study participant.

Outlook

In order to fulfill the above mentioned requirements for follow-up studies, our group has developed a MR compatible dynamometer for the measurement of muscular force during isometric contractions of the plantarflexor muscles against a pedal which allows conducting HE as well as LE protocols. Visual feedback of the measured force to the subject under investigation is granted, which is expected to increase the compliance of study participants with the protocol requirements. We are looking forward on testing the hypotheses developed above with our new ergometer, paying attention to the requirements identified by our preliminary study.

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